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Alternating Access and a Pore-Loop Structure in the Na⁺-Citrate Transporter CitS of *Klebsiella pneumoniae**

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CitS of *Klebsiella pneumoniae* is a secondary transporter that transports citrate in symport with 2 Na⁺ ions. Reaction of Cys-398 and Cys-414, which are located in a cytoplasmic loop of the protein that is believed to be involved in catalysis, with thiol reagents resulted in significant inhibition of uptake activity. The reactivity of the two residues was determined in single Cys mutants in different catalytic states of the transporter and from both sides of the membrane. The single Cys mutants were shown to have the same transport stoichiometry as wild type CitS, but the C398S mutation was responsible for a 10-fold loss of affinity for Na⁺. Both cysteine residues were accessible from the periplasmic as well as from the cytoplasmic side of the membrane by the membrane-impermeable thiol reagent [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) suggesting that the residues are part of the translocation site. Binding of citrate to the outward facing binding site of the transporter resulted in partial protection against inactivation by *N*-ethylmaleimide, whereas binding to the inward facing binding site resulted in essentially complete protection. A 10-fold higher concentration of citrate was required at the cytoplasmic rather than at the periplasmic side of the membrane to promote protection. Only marginal effects of citrate binding were seen on reactivity with MTSET. Binding of Na⁺ at the periplasmic side of the transporter protected both Cys-398 and Cys-414 against reaction with the thiol reagents, whereas binding at the cytoplasmic side was less effective and discriminated between Cys-398 and Cys-414. A model is presented in which part of the cytoplasmic loop containing Cys-398 and Cys-414 folds back into the translocation pore as a pore-loop structure. The loop protrudes into the pore beyond the citrate-binding site that is situated at the membrane-cytoplasm interface.

The secondary citrate transporter CitS of *Klebsiella pneumoniae* is a member of the bacterial 2-hydroxycarboxylate transporter (2HCT)¹ family. Members of the 2HCT family

transport substrates that contain the 2-hydroxycarboxylate motif, as in citrate, malate, lactate, etc. (1). The family contains H⁺ and Na⁺ symporters that couple the translocation of the substrate to the co-ion as well as precursor/product exchangers that couple the uptake of the substrate to the excretion of a metabolic end product (2, 3). CitS is a Na⁺ symporter. It obligatorily couples the translocation of the divalent citrate anion (Hcit²⁻) to the translocation of two sodium ions and one proton (4, 5). The CitS protein is an integral membrane protein containing 446 amino acid residues (47.5 kDa). The protein is believed to consist of a bundle of 11 hydrophobic transmembrane segments (TMSs) with the N and C terminus located in the cytoplasm and periplasm, respectively (Fig. 1) (6–9). An additional hydrophobic segment, predicted to be transmembrane as well, was shown to reside in the periplasm between TMSs V and VI. The segment, termed Vb, and a stretch of about 40 residues in the C-terminal part of the protein (region Xa) are the two most conserved regions in the 2HCT family. Evidence is accumulating that region Xa is part of the translocation site in the protein. Conserved residue Arg-428 in TMS XI situated close to the membrane/cytoplasm interface is believed to bind one of the carboxylate groups of the substrate (10, 11).

Recent crystal structures of the LacY and GlpT proteins, both secondary transporters from the major facilitator superfamily, support the alternating access model for the translocation mechanism of secondary transporters (12, 13). In this model, the proteins contain single binding sites for substrate and co-ions that alternately are exposed to the two sides of the membrane during the catalytic cycle. Coupling between the co-ion and substrate fluxes is warranted by the condition that only the transporters with the free and with the fully occupied binding sites (“unloaded” and “fully loaded” states, respectively) can reorient the binding sites, whereas the conformational change is blocked when co-ion or substrate is bound. An important consequence of the alternating access model is that residues, which are part of the translocation site and which in topology models of the transporter reside in the membrane, are in contact with the water phase. In a number of recent studies this property was explored to identify (part of) the translocation site by identifying residues in transmembrane segments that were accessible to small water-soluble methanethiosulfonate (MTS) derivatives by using cysteine-scanning mutagenesis (14–16). A related consequence is that certain of these residues are expected to be accessible from both sides of the membrane when the protein can freely reorient the binding sites. Surprisingly, in a number of cases, this was observed for residues in loop regions, rather than TMSs, which led to the suggestion of pore-loop structures or re-entrant loops in secondary transport-

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¹ The abbreviations used are: 2HCT, 2-hydroxycarboxylate transporter; TMS, transmembrane segment; RSO, right side out; ISO, inside-out; PMS, phenazine methosulfate; NEM, *N*-ethylmaleimide; AmdIS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; MTSET, [2-(tri-

methylammonium)ethyl] methanethiosulfonate bromide; FM, fluorescein 5-maleimide; MTS, methanethiosulfonate; DTT, dithiothreitol.

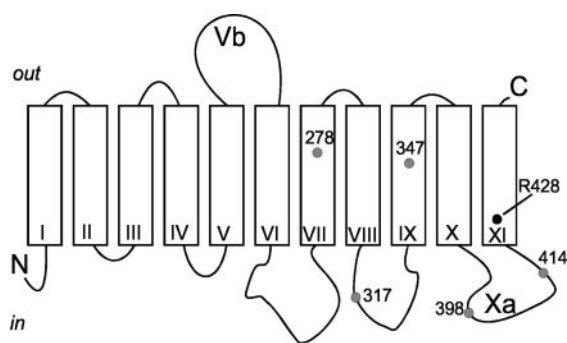


FIG. 1. **Membrane topology model of CitS.** Boxes represent putative transmembrane segments. The length of the loops correlates more or less with the number of residues in the loops. Segment Vb corresponds to a hydrophobic region that was predicted to be transmembrane. Loop Xa is one of the best conserved regions in the 2HCT family. Numbers indicate the positions of the five endogenous cysteine residues. Arg-428 (R428) in TMS XI is involved in substrate binding.

ers (17–22). Pore-loop structures are loop regions that fold back between the TMSs and are commonly observed in channel proteins where they function as selectivity filters (23, 24). Well studied examples are the bacterial and neuronal glutamate transporters, GltT and GltT-1, in which pore-loops were shown to play an important role in the translocation event (17, 18, 25, 26). Pore-loop structures are not observed in the LacY and GltP structures, and it is unknown whether or not they represent a different translocation mechanism.

The CitS protein contains five cysteine residues, all located in the C-terminal part of the transporter. Cys-398 and Cys-414 are found in region Xa in the cytoplasmic loop between TMS X and TMS XI (Fig. 1). Recently, we showed that alkylation of the two cysteine residues by *N*-ethylmaleimide (NEM) resulted in loss of activity, in line with the importance of region Xa in catalysis (27). Most importantly, inactivation was also observed with the membrane-impermeable thiol reagent [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) added at the periplasmic side of the membrane revealing an access pathway for the reagent between the periplasm and the residues, possibly by formation of a pore-loop structure by the cytoplasmic loop. The access pathway was blocked in the presence of the co-ion Na^+ indicating that the region is sensitive to translocation-linked conformational changes of the transporter. Here we study in detail the accessibility of Cys-398 and Cys-414 in single cysteine mutants of CitS in different catalytic states of the transporter. We probed the two cysteine residues by different sulfhydryl reagents and from both sides of the membrane. The catalytic state of the transporter was modulated through the presence and absence of citrate, Na^+ , or both. It follows that both positions are accessible from either side of the membrane and that the accessibility is different in the two orientations of the transporter. The results are consistent with a pore-loop structure formed by part of region Xa.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and CitS Constructs—*Escherichia coli* strains DH5 α and ECOMUT2 (28) were routinely grown in Luria Bertani broth (LB) medium at 37 °C under continuous shaking at 150 rpm. When appropriate, the antibiotics ampicillin and chloramphenicol were added at a final concentration of 50 and 30 $\mu\text{g}/\text{ml}$, respectively. All genetic manipulations were done in *E. coli* DH5 α whereas the CitS protein was expressed in *E. coli* ECOMUT2 harboring plasmid pBADCitS and derivatives. Plasmid pBADCitS is based on vector pBAD24 (Invitrogen) and codes for the wild type CitS protein with an N-terminal His tag. Expression of the gene is under control of the arabinose promoter (27). Expression of CitS was induced by adding 0.1% arabinose when the absorbance of the culture measured at 660 nm (A_{660}) reached a value of 0.6.

Cysteine mutants of CitS were constructed by PCR using the QuickChange™ Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). Plasmid pHisCitS is based on the pBluescript vector (Stratagene) and was used as the template (7). All mutants were sequenced (Baseclear, Leiden, The Netherlands) to confirm the presence of the desired mutations and cloned into the pBAD24 vector as described before (27).

Preparation of Right Side Out and Inside Out Membrane Vesicles—*E. coli* ECOMUT2-expressing CitS variants were harvested from a 1-liter culture by centrifugation at 10,000 $\times g$ for 10 min at 4 °C. Right side out (RSO) membrane vesicles were prepared by the osmotic lysis procedure as described (29).

Inside out (ISO) membranes were prepared by washing the cells once with 10 mM Tris-HCl buffer, pH 8, followed by resuspending in 10 ml of 50 mM KP_i buffer, pH 7, containing 2 mM MgSO_4 and 10 $\mu\text{g}/\text{ml}$ deoxyribonuclease. The suspension was passed once through a French press cell operated at 13,000 pounds/square inch. Cell debris and unbroken cells were removed by centrifugation at 8000 rpm for 10 min at 4 °C in a Beckman SS34 rotor. Membranes were collected by ultracentrifugation for 25 min at 80,000 rpm at 4 °C in a Beckman TLA 100.4 rotor and washed once with 50 mM KP_i, pH 7.

RSO and ISO membranes were resuspended in low Na^+ 50 mM KP_i, pH 7.0, rapidly frozen, and stored in liquid nitrogen. Membrane protein concentration was determined by the DC Protein Assay Kit (Bio-Rad).

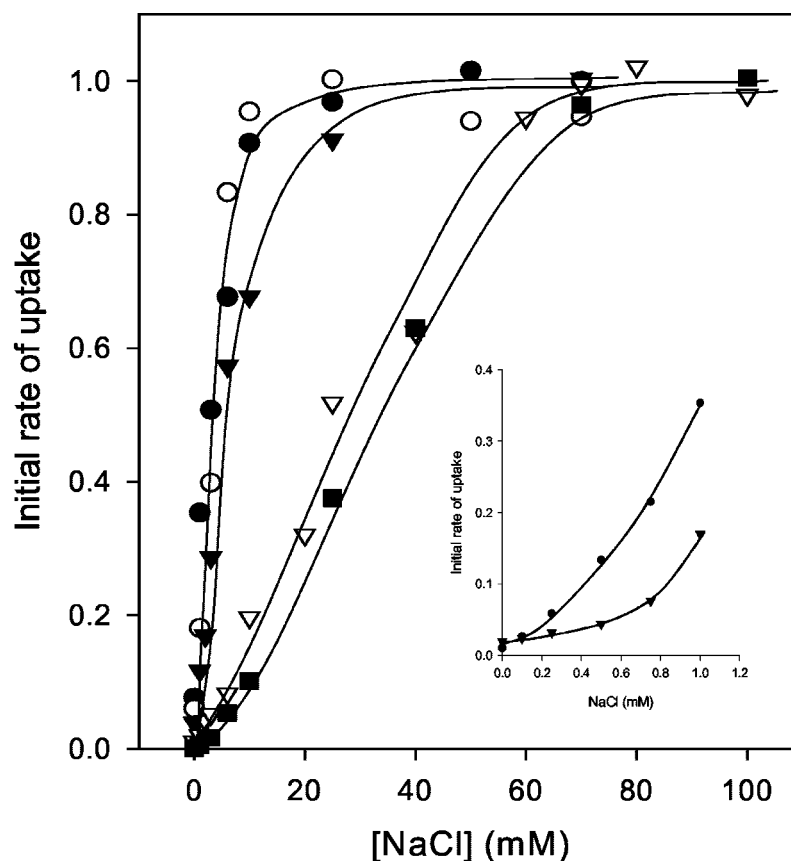
Partial Purification of CitS Using Nickel-Nitrilotriacetic Acid Affinity Chromatography—ISO membranes (4 mg/ml) containing His-tagged CitS derivatives were solubilized in 50 mM KP_i, pH 8, 400 mM NaCl, 20% glycerol, and 1% Triton X-100. The solution was left on ice for 30 min. Undissolved material was removed by ultracentrifugation at 80,000 rpm for 25 min at 4 °C. The supernatant was mixed with Ni^{2+} -nitrilotriacetic acid resin (100- μl bed volume per 10 mg of protein), equilibrated in 50 mM potassium phosphate pH 8.0, 600 mM KCl, 10% glycerol, 0.1% Triton X-100, 10 mM imidazole and incubated for 1 h at 4 °C under continuous shaking. After incubation, the resin was pelleted down by pulse centrifugation, and the supernatant was removed. The pellet was washed with 10 volumes of equilibration buffer containing 300 mM KCl and 40 mM imidazole. The protein was eluted with 1 bed volume of the same washing buffer but containing 50 mM potassium phosphate, pH 7.0, and 150 mM imidazole and stored at –20 °C.

Transport Assay in RSO Membranes—Uptake was measured by the rapid filtration method. RSO membranes were energized using the potassium ascorbate/phenazine methosulfate (PMS) electron donor system (30). Membranes were diluted to a final concentration of 0.5 mg/ml into 50 mM KP_i, pH 6.0, containing 70 mM Na^+ unless stated otherwise, in a total volume of 100 μl at 30 °C. Under a constant flow of water-saturated air, and while stirring magnetically, 10 mM potassium ascorbate and 100 μM PMS (final concentration) were added, and the proton motive force was allowed to develop for 2 min. Then, [$1,5\text{-}^{14}\text{C}$]citrate (114 mCi/mmol Amersham Biosciences) was added to a final concentration of 4.4 μM . The uptake was stopped by the addition of 2 ml of ice-cold 0.1 M LiCl, followed by immediate filtration over cellulose nitrate filters (0.45 μm , pore size). The filters were washed once with 2 ml of the 0.1 M LiCl solution and assayed for radioactivity. The background was estimated by adding the radiolabeled substrate to the vesicle suspension after the addition of 2 ml of ice-cold LiCl, immediately followed by filtering. When indicated, experiments were carried out in low Na^+ KP_i buffer that contains at most 0.005% of Na^+ , whereas standard KP_i buffers may contain up to 0.3%.

Treatment of RSO and ISO Membranes with Thiol Reagents—Stock solutions of NEM, MTSET, and Amdis were prepared freshly in water. RSO membranes at a concentration of 1 mg/ml and ISO membranes at a concentration of 2 mg/ml were treated with the thiol reagents in 50 mM KP_i, pH 7.0, at 20 °C. The treatment was stopped by addition of an equal concentration of DTT in case of NEM and Amdis or reduced glutathione (GSH) in case of MTSET. The effect of Na^+ on the treatment was measured by adding mixtures of NaCl and KCl yielding the same concentration of chloride. The effect of citrate on the treatment was measured by adding citrate from a 0.5 M stock solution adjusted to pH 6.0.

After the treatment, RSO membranes were diluted twice into 50 mM KP_i, pH 5.0. If necessary, NaCl and KCl were added to make the Na^+ concentrations 70 mM. The resulting suspension was pH 6 and was immediately used to measure uptake activity. The presence of DTT or GSH did not affect the uptake rate in the control experiments. Following the treatment in the presence of citrate, the membranes were washed twice with 50 mM KP_i, pH 6.0, by centrifugation for 20 min in a Beckman Optima™ TLX ultracentrifuge in a TLA100.2 rotor to remove citrate. Membranes were resuspended in 50 mM KP_i, pH 6.0, containing 70 mM Na^+ and assayed for uptake activity. Control exper-

FIG. 2. The initial rate of citrate uptake as a function of the Na^+ concentration in RSO membranes containing wild type CitS (CCCCC, ●) and the CitS cysteine mutants, SSSCC (○), SSSCS (▼), SSSSC (▽), and SSSSS (■). The rate was plotted as the fraction of the rate measured at saturated Na^+ concentrations of 4.2 (CCCCC), 1.5 (SSSCC), 3.2 (SSSCS), 2.4 (SSSSC), and 2.0 (SSSSS) nmol/min per mg of protein. *Inset*, initial rate of uptake catalyzed by wild type CitS (CCCCC, ●) and the SSSCS mutant (▼) in the concentration range up to 1 mM Na^+ .



iment showed that the washing procedure resulted in a loss of 10–15% of the uptake activity.

After the treatment of ISO membranes, CitS derivatives were partially purified using nickel-nitrilotriacetic acid affinity chromatography as described above and, subsequently, labeled with 0.1 mM fluorescein 5-maleimide (FM) in elution buffer for 5 min at room temperature. The reaction was quenched with 0.5 mM DTT, and after addition of SDS sample buffer, 25- μ l samples were run on a 12% SDS-PAGE gel. Fluorescence of proteins labeled with FM was visualized on a Lumi-Imager F1 imager (Roche Diagnostics) by irradiation with UV light using a 520-nm filter. All samples containing FM were kept out of bright light until the gel was exposed. After exposure, the gel was stained with Coomassie Brilliant Blue to compare the protein levels of CitS. Quantification was done using the LumiAnalyst 3.1 software package supplied by Roche Diagnostics.

Materials—2-(Trimethylammonium) ethyl methanethiosulfonate bromide (MTSET) was purchased from Anatrace Inc. (Ohio). NEM was purchased from Sigma, and AmdIS and FM were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Low Na^+ potassium buffer (KH_2PO_4) was purchased from Merck.

RESULTS

Activity of Single Cysteine Mutants SSSCS and SSSSC—Wild type CitS transports citrate obligatorily coupled to the translocation of two Na^+ ions (4). The rate of uptake in RSO membranes prepared from *E. coli* cells expressing CitS and energized by the artificial electron donor system ascorbate/PMS increased sigmoidally with increasing Na^+ concentration. Half of the maximal rate was obtained at a Na^+ concentration of 3 mM (27) (Fig. 2, ●). The shape of the curve was not significantly different for a mutant of CitS in which three out of the five endogenous cysteine residues were replaced by serines (mutant SSSCC, ○). In addition, removing Cys-414, located in the C-terminal loop of CitS (see Fig. 1), resulted in a slightly lower affinity for Na^+ (mutant SSSCS, ▼) without affecting the shape of the curve (Fig. 2, *inset*). Half of the maximal rate was obtained at a concentration of 6 mM Na^+ . The low residual uptake rate observed in the absence of added Na^+ (about 2% of

the saturated rate; Fig. 2, *inset*) is due to low Na^+ ion contaminations of the buffer system. A more drastic change in affinity was observed when in addition to the three N-terminal cysteine residues, Cys-398 was replaced by serine (mutant SSSSC, Fig. 2, ▽). The apparent affinity for Na^+ dropped to 28 mM, and again, the sigmoidal increase of the rate with the Na^+ concentration was retained. A similar reduction in affinity for Na^+ was observed for the Cys-less mutant (SSSSS, Fig. 2, ■) suggesting that the C398S mutation in the SSSSC and SSSSS mutants was responsible for the observed effect. The small difference in affinity between the SSSSC and SSSSS mutants is consistent with the observed difference between the SSSCC and SSSCS mutants and suggests that the C414S mutation lowers the affinity for Na^+ independently of the effect of the C398S mutation.

Finally, in the single cysteine mutants SSSCS and SSSSC, the mechanism of citrate translocation is the same as observed for wild type CitS. Uptake is strictly coupled to the uptake of Na^+ , and two Na^+ are required for one translocation cycle.

Accessibility of Cys-398 and Cys-414 in the Unloaded State of the Transporter—Treatment of RSO membrane vesicles containing CitS mutant SSSCS with the membrane-permeable maleimide NEM at a concentration of 2 mM for increasing periods of time resulted in inhibition of the initial rate of citrate uptake down to a residual activity of about 10% (Fig. 3A, ●). The half-time of inactivation was 8 min. Treatment of the same membranes with the membrane-impermeable and more bulky maleimide 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AmdIS) did not result in significant inactivation under the same conditions (Fig. 3A, ○). In contrast, the membrane-impermeable but much smaller methanethiosulfonate MTSET was very efficient in inactivating the SSSCS mutant. At the same concentration, the half-time of inactivation was about 10 times shorter for MTSET than observed for NEM (Fig. 3A, ▼).

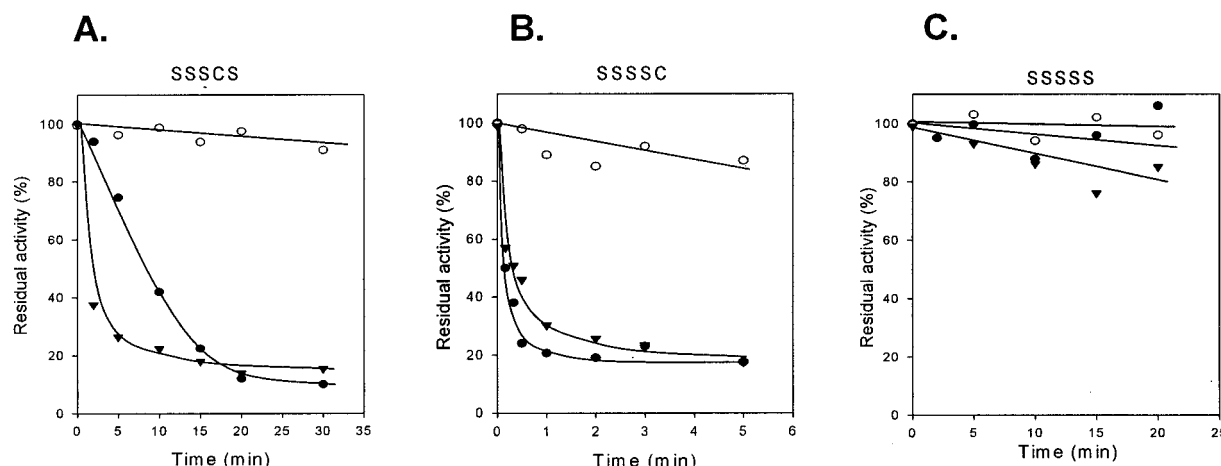


FIG. 3. Residual activity of citrate uptake after treatment of RSO membranes containing the SSSCS (A), SSSSC (B), and SSSSS (C) mutant with NEM (●), AmdIS (○), and MTSET (▼). RSO membranes were incubated for the indicated time with 0.25 mM AmdIS (SSSCS, SSSSC, SSSSS), 2 mM MTSET (SSSCS, SSSSS), 0.5 mM MTSET (SSSSC), 2 mM NEM (SSSCS), 1 mM NEM (SSSSS), and 0.2 mM NEM (SSSSC). Initial rates were expressed as the percentage of the initial rate catalyzed by untreated membranes.

It should be noted that the reagents only had a marginal effect on the uptake activity of RSO membrane vesicles containing the Cys-less CitS mutant SSSSS (Fig. 3C). Qualitatively, the results were the same for the reactivity of Cys-414 in mutant SSSSC (Fig. 3B). Both NEM and MTSET inactivated the transporter, whereas AmdIS did not. Quantitatively, the rates of inactivation were much faster than observed for the SSSCS mutant, with half-times below 20 s at concentrations of the thiol reagents that were lower than used to inactivate SSSCS. Both mutants showed a residual activity between 10 and 20% after reaction with NEM and MTSET. It follows that Cys-398 and Cys-414 are both accessible to NEM and MTSET added at the periplasmic side of the membrane. The different rates of reaction may reflect differences in accessibility, different reactivities of the two thiols, or both.

Membrane vesicles with an inverted orientation (inside out, ISO) were used to determine the accessibility of Cys-398 and Cys-414 with thiol reagents NEM, AmdIS, and MTSET from the cytoplasmic side of the membrane. Mutants SSSCS and SSSSC purified from ISO membranes in detergent solution were visualized by labeling of the single cysteine residues with the fluorescent maleimide FM, followed by SDS-PAGE and fluorescence imaging of the gel (Fig. 4, A and B, lanes 1). No fluorescent protein band was observed for the Cys-less CitS (SSSSS; Fig. 4C). Pretreatment of purified SSSCS and SSSSC with NEM (Fig. 4, A and B, lanes 3), AmdIS (lanes 5), and MTSET (lanes 7) prevented subsequent labeling with FM, showing that all molecules were accessible to the reagents in the solubilized state.

Treatment of ISO membranes containing the SSSCS or SSSSC mutants before purification with 2 mM NEM for 20 min (SSSCS) or 0.5 mM NEM for 5 min (SSSSC) under the same conditions as the RSO membranes above resulted in a >90% decrease of the labeling with FM after purification of the proteins (Fig. 4, A and B, lanes 2). As expected, membrane-permeable NEM was equally capable of reacting with both cysteine residues in RSO and ISO membranes. Remarkably, treatment of the membranes with 0.25 mM AmdIS for 20 (SSSCS) or 5 min (SSSSC) significantly reduced labeling with FM indicating that, in contrast to RSO membranes, in ISO membranes Cys-398 and Cys-414 are accessible to AmdIS (Fig. 4, A and B, lanes 4). Treatment of the ISO membranes containing SSSCS and SSSSC with MTSET at a concentration and for a time that resulted in 90% inhibition in RSO vesicles almost completely blocked further labeling with FM (Fig. 4, A and B, lanes 6),

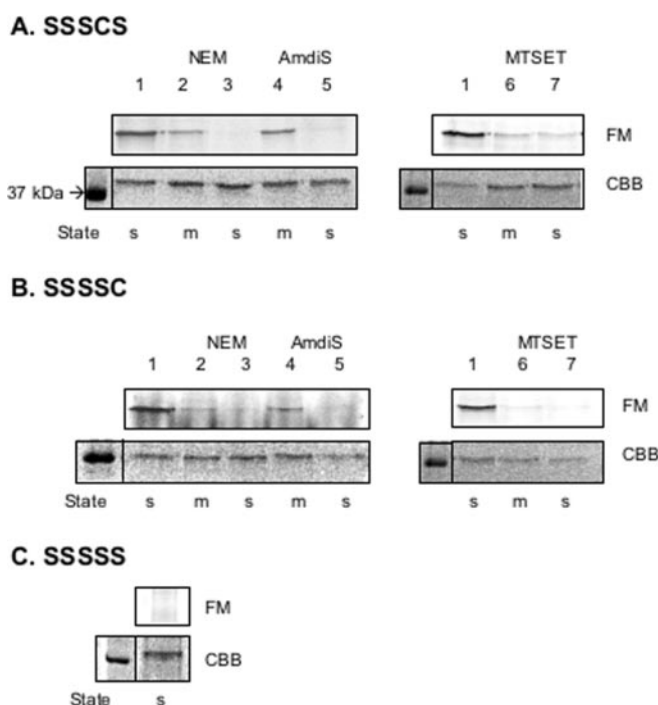
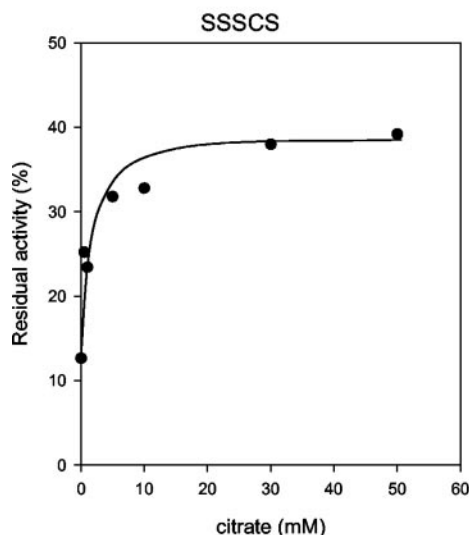


FIG. 4. Labeling of the SSSCS (A), SSSSC (B), and the SSSSS (C) mutants with FM. ISO membranes were not further treated (lanes 1) or pretreated with NEM (lanes 2 and 3), AmdIS (lanes 4 and 5), and MTSET (lanes 6 and 7) in the presence (s) or absence (m) of 1% of the detergent Triton X-100. The conditions of the pretreatment with the thiol reagents were 2 mM NEM, 0.25 mM AmdIS, and 2 mM MTSET for 20 min (A), and 0.5 mM NEM, 0.25 mM AmdIS, and 0.5 mM MTSET for 5 min (B). After the treatment, the mutants were purified and treated with 0.1 mM FM for 5 min. The top rows (FM) show the fluorescence image of the gel, and the bottom rows show the gel after Coomassie Brilliant Blue (CBB) staining. Shown on the left is the 37-kDa marker.

showing that MTSET accesses the cysteine residues in both RSO and ISO membranes.

In conclusion, in the unloaded state of the transporter, *i.e.* in the absence of citrate and Na^+ , both cysteine residues in region Xa of CitS are accessible from the periplasmic as well as the cytoplasmic side of the membrane for membrane-permeable NEM and membrane-impermeable MTSET. Access for the more bulky, impermeable AmdIS is restricted from the periplasmic side but not from the cytoplasmic side.

A. RSO



B. ISO

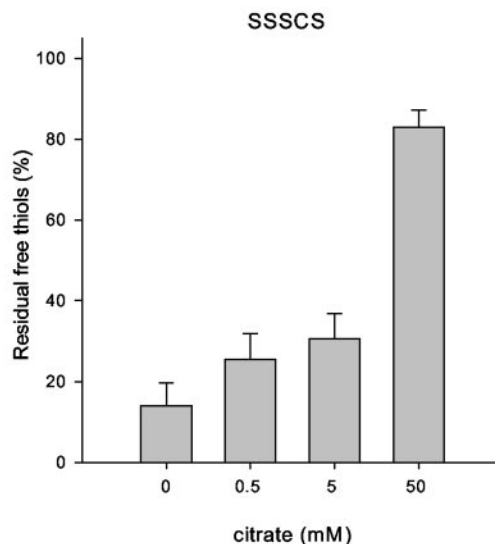


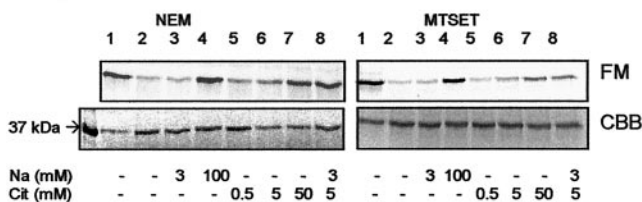
FIG. 5. **Protection by citrate of the SSSCS mutant against NEM inactivation.** Residual activity after treatment of RSO membranes (A) and residual free thiols after treatment of ISO membranes (B) containing the SSSCS mutant with 2 mM NEM for 20 min in the presence of the indicated concentrations of citrate. Residual activity and residual free thiols were expressed as the percentage of the activity and free thiols of untreated membranes. The bars in B represent average and standard deviation obtained from two or three independent measurements. A typical experiment is shown in Fig. 6A, left panel, lanes 1 and 5–7.

Accessibility of Cys-398 and Cys-414 in the Citrate-bound State of the Transporter—In RSO membranes, containing the SSSCS mutant the presence of citrate resulted in partial protection of CitS against NEM inactivation (Fig. 5A). The membranes were treated with 2 mM of NEM for 20 min, which just about resulted in maximal inactivation in the absence of citrate (see Fig. 3A). Under these conditions, the residual activity increased from 14% in the absence of citrate to 38% at 50 mM citrate. The lack of change in residual activity above citrate concentrations of 10 mM indicated that the thiol did react with NEM in the citrate-bound state but at a slower rate than in the free state. A concentration of citrate below 1 mM was enough to give half of the maximal difference in residual activity. The experiment shows that CitS binds citrate and that Cys-398 is accessible to NEM both in the free and in the citrate-bound state.

In ISO membranes, using the same concentration of NEM for the same amount of time, the presence of up to 5 mM citrate had little effect on the labeling by NEM (Fig. 6A, lanes 2, 5, and 6). However, at 50 mM citrate significant inhibition of NEM labeling was observed. Quantification of the fluorescence intensity showed that the residual free thiols after NEM treatment at 50 mM citrate amounted up to 80% and that the concentration to obtain half of the maximal protection was between 5 and 50 mM (Fig. 5B), which is at least 10 times higher than observed for the RSO membranes. The latter is explained by a higher reactivity of the thiol or a lower affinity for citrate at the cytoplasmic side of the membrane as compared with the periplasmic side. At any rate, citrate protects Cys-398 more efficiently in the inward facing than in the outward facing binding site.

The presence of citrate had small but significant effects on the reaction of Cys-398 with membrane-impermeable MTSET. In RSO membranes, the residual activity after treatment with 2 mM MTSET for 5 min increased from 21 to 36% when 50 mM citrate was present during the treatment (Table I). After the same treatment of ISO membranes in the presence of increasing concentrations of citrate, the percentage of free thiols changed from 13 to 28% (Fig. 6A, lanes 2, 5, and 7; Table I). Apparently, in the citrate-bound state, the accessibility of Cys-398 in the SSSCS mutant for MTSET was only marginally

A. SSSCS



B. SSSSC

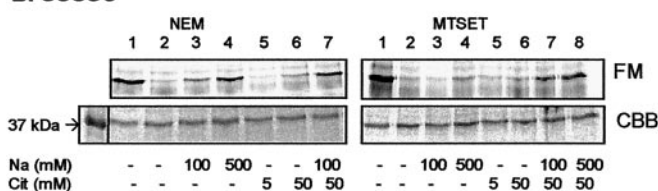


FIG. 6. **Effect of Na⁺ and citrate on the labeling of SSSCS (A) and SSSSC (B) in ISO membranes with NEM and MTSET.** ISO membranes were not further treated (lanes 1) or pretreated with NEM (left panels, lanes 2–8) and MTSET (right panels, lanes 2–8) in the presence of the indicated concentrations of Na⁺ and citrate. The conditions of the treatment were 2 mM NEM for 20 min and 2 mM MTSET for 5 min (A) and 0.5 mM NEM and 0.5 mM MTSET for 1 min (B). After the treatment, the mutants were purified and treated with 0.1 mM FM for 5 min. The top rows (FM) show the fluorescence image of the gel, and the bottom rows show the gel after Coomassie Brilliant Blue (CBB) staining. Shown on the left is the 37-kDa marker.

affected in either orientation of the transporter. Similarly, only small effects were observed on the accessibility of Cys-414 in the SSSSC mutant. In RSO membranes, no significant effect on the residual activity in the presence of citrate was observed, both against NEM and against MTSET (Table I), whereas in ISO membranes the protection was minimal (Table I and Fig. 6B, lanes 2, 5, and 6). Some protection against both NEM and MTSET labeling was observed at 50 mM citrate and not at 5 mM citrate, as observed for Cys-398 mutant (Fig. 4B).

Accessibility of Cys-398 and Cys-414 in the Na⁺-bound State of the Transporter—It was demonstrated previously (27) that

TABLE I

Effect of citrate on the reaction of single Cys mutants SSSCS and SSSSC with NEM and MTSET in RSO and ISO membranes

RSO and ISO membranes were treated with 2 mM NEM for 20 min or 2 mM MTSET for 5 min (SSSCS) and with 0.5 mM NEM and 0.5 mM MTSET for 1 min (SSSSC). The indicated values give the residual uptake activity in RSO membranes or residual free thiols in ISO membranes as the percentage of an untreated sample. The average and standard deviations of 2–3 independent measurements are reported.

	SSSCS, citrate		SSSSC, citrate	
	0	50	0	50
	mM		mM	
NEM				
RSO	14 ± 2	38 ± 3	18 ± 2	28 ± 4
ISO	18 ± 3	80 ± 4	10 ± 3	22 ± 2
MTSET				
RSO	21 ± 3	36 ± 5	35 ± 5	40 ± 5
ISO	13 ± 3	28 ± 4	10 ± 4	18 ± 4

the presence of Na⁺ ions protects the wild type CitS protein in RSO membranes against inactivation by NEM. Similarly, mutants SSSCS and SSSSC in RSO membranes were essentially fully protected against inactivation by both NEM and MTSET in the Na⁺-bound state. Under conditions where the thiol reagents inactivated the mutant transporters to maximal levels (10–20% residual activity) in Na⁺-free buffers, the residual activity was over 80% in the presence of saturating concentrations of Na⁺ (Fig. 7A). In agreement with the lower affinity for Na⁺ (Fig. 2), a higher concentration was needed to obtain maximal protection of the SSSSC mutant than observed for the SSSCS mutant (70 and 500 mM, respectively). In the presence of Na⁺ concentrations of up to 70 mM, no protection was observed in case of the SSSSC mutant (not shown). In conclusion, in RSO membranes, the presence of sodium ions clearly protects both single Cys mutants against reacting with both the membrane-permeable and -impermeable cysteine reagents.

In ISO membranes, the presence of 3 mM Na⁺ did not affect the labeling of mutant SSSCS with NEM, but increasing the concentration up to 100 mM essentially completely prevented labeling (Fig. 6A, lanes 3 and 4). Quantification of the fluorescence revealed over 90% residual free thiols following the latter condition (Fig. 7B) which corresponds to a similar level of protection as observed in RSO membranes. With MTSET as the modifying reagent, results were similar, but the maximal level of protection was significantly lower (~50%) than observed in the RSO membranes (Fig. 6A lanes 3 and 4). Cys-414 in mutant SSSSC was significantly less well protected by Na⁺ against both reagents in ISO membranes than in RSO membranes. At 500 mM Na⁺, the residual free thiols amounted up to ~55% after NEM treatment, whereas hardly any effect could be observed after MTSET treatment (Fig. 6B, lanes 1, 3, and 4, and Fig. 7B).

Accessibility of Cys-398 and Cys-414 in Fully Loaded State of the Transporter—RSO membranes containing the SSSCS mutant were treated with 2 mM NEM for 20 min in the presence of 3 mM Na⁺, which is too low to affect significantly the inactivation rate. The residual activity was 28% (Table II). In parallel, the membranes were treated the same way in the presence of 50 mM citrate instead of 3 mM Na⁺. This concentration of citrate results in the maximal protection by substrate (see Fig. 5A), and the residual activity in this particular experiment was 38%. Surprisingly, upon treatment with NEM in the presence of both 3 mM Na⁺ and 50 mM citrate, which brings the transporter in the translocation competent state, the transporter was essentially completely protected against inactivation under the conditions of the treatment (Table II). Apparently, when the transporter is in the fully loaded state, Cys-398 is much less accessible from either side of the membrane by

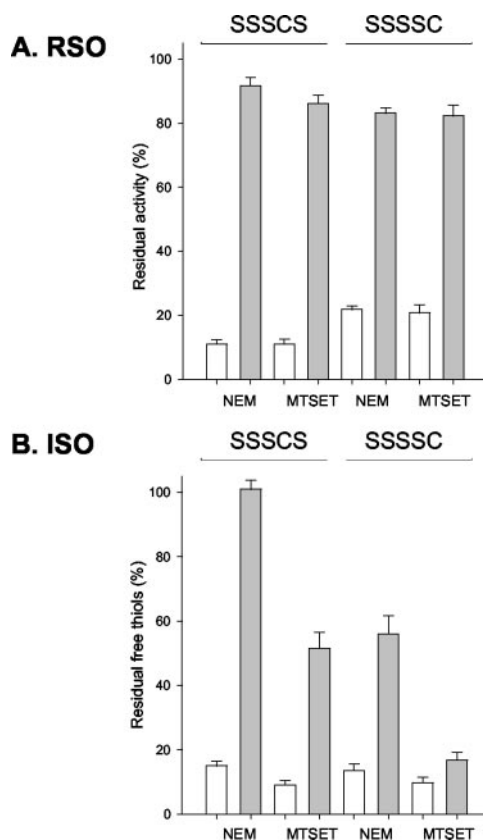


FIG. 7. Effect of Na⁺ on the reaction of the cysteine mutants with NEM and MTSET. RSO (A) and ISO (B) membranes were treated with 2 mM NEM for 20 min or 2 mM MTSET for 5 min (SSSCS), and with 0.5 mM NEM and 0.5 mM MTSET for 1 min (SSSSC) in the absence (white bars) or presence (gray bars) of 100 (SSSCS) or 500 mM (SSSSC) Na⁺. Residual activity and residual free thiols were shown as the percentage of an untreated sample. The bars represent average and standard deviation obtained from 2 to 3 independent measurements.

TABLE II

Effect of citrate and sodium on the inactivation of mutants SSSCS and SSSSC with NEM and MTSET in RSO membrane vesicles

RSO membranes were treated with 2 mM NEM for 20 min or 2 mM MTSET for 5 min (SSSCS) and with 0.5 mM NEM and 0.5 mM MTSET for 1 min (SSSSC). The indicated values give the residual uptake activity in RSO membranes as the percentage of an untreated sample. The average and standard deviations of 2–3 independent measurements are reported.

Mutant	Reagent	Addition	Residual activity
			%
SSSCS	NEM	3 mM Na ⁺	28 ± 5
		50 mM citrate	38 ± 3
		Both	92 ± 3
	MTSET	3 mM Na ⁺	21 ± 3
		50 mM citrate	36 ± 5
		Both	88 ± 4
SSSSC	NEM	70 mM Na ⁺	38 ± 3
		50 mM citrate	28 ± 4
		Both	82 ± 4
	MTSET	70 mM Na ⁺	31 ± 3
		50 mM citrate	40 ± 5
		Both	78 ± 5

membrane-permeable NEM. By using a slightly different set of concentrations, the same effect was observed for the SSSSC mutant and with MTSET as the thiol reagent (Table II).

In ISO membranes, a concentration of 3 mM Na⁺ or 5 mM citrate hardly affected the labeling of Cys-398 in mutant SSSCS by NEM. However, the combination of the two resulted in almost complete protection (compare lanes 3, 6, and 8 in Fig. 6A). Similarly, 100 mM Na⁺ and 50 mM citrate protected Cys-

414 in the SSSSC mutant much better in combination than separately, even though the protection was not complete (compare lanes 3, 6, and 7 in Fig. 6B). In summary, in the fully loaded state Cys-398 and Cys-414 appear to be the least accessible from either side of the membrane.

DISCUSSION

In this study, we investigated the accessibility of residues Cys-398 and Cys-414 in region Xa of the Na⁺ citrate transporter CitS of *K. pneumoniae* in different catalytic states of the transporter. Accessibility was studied in single Cys mutants in which the other four endogenous cysteine residues were mutated to Ser (SSSCS and SSSSC). Both single Cys mutants were active in citrate transport but the mutant SSSSC had a 10-fold lower apparent affinity for Na⁺ ions (Fig. 2). The lowered affinity was observed in mutants SSSSC and SSSSS but not in mutants SSSCC and SSSCS, which identifies C398S as the responsible mutation. In all mutants tested, the lack of uptake in the absence of Na⁺ and the sigmoidal increase of the rate of uptake with increasing Na⁺ concentration was retained. Therefore, the mechanism of citrate translocation is the same for the mutants and wild type CitS; transport of citrate is strictly coupled to translocation of two Na⁺. Mutation C398S that results in reduced affinity for Na⁺ is the first mutation in CitS or any other member of the 2HCT family that affects the interaction of the transporter with the co-ion. The residue corresponding to Arg-428 in region Xa of CitS is known to interact with the substrate (11, 31).

Previous studies of the SSSCC mutant of CitS and cysteine mutants of the homologous transporter CimH of *Bacillus subtilis* showed, surprisingly, that residues in region Xa were accessible for membrane-impermeable MTS derivatives when presented at the periplasmic side of the membrane (11, 27). The MTS derivatives are similar in size to citrate (32), and it was suggested that access from the periplasmic side of the membrane might follow (part of) the substrate translocation pathway (also see Ref. 33). Access of residues in a cytoplasmic loop from the periplasm was explained by part of the loop folding back into the translocation pore (a pore-loop structure) or by a water-filled pore, which would extend all the way to the membrane/cytoplasm interface where the cytoplasmic loop would form the base of the pore. The present study shows that both cysteine residues in region Xa of CitS that are 16 positions apart in the primary structure are accessible for MTSET from the periplasmic side of the membrane. Moreover, binding of citrate to the outwardly exposed binding site affected the reactivity of the two residues with MTSET only marginally (Cys-398) or not significantly (Cys-414) suggesting that the accessibility was not seriously restricted (Table I). Although only a three-dimensional structure at atomic resolution will provide the final answer, we feel that these characteristics are more in line with a pore-loop model as detailed at the end of the "Discussion" than with a water-filled pore model. Most important, both residues were equally well accessible from the cytoplasmic side of the membrane. Access of the same site in a transporter from both sides of the membrane is well explained by the alternating access model of the translocation mechanism. Residues that are part of the translocation site are accessible from the two faces of the membrane under conditions when the transporter can freely reorient the binding sites. Accessibility from both sides corresponds to accessibility in two different states of the protein. Access of residues in a cytoplasmic loop from both sides of the membrane was reported before for the glutamate transporters GltT of *Bacillus stearothermophilus* and GLT-1 from the central nervous system (17, 34) and the melibiose transporter MelB of *E. coli* (15). In the case of GLT-1,

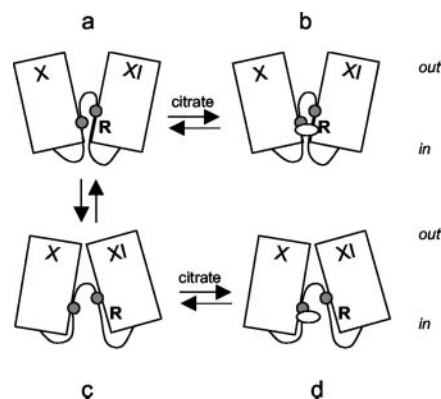


FIG. 8. Model of the translocation site of CitS. Shown are conformational changes in the part of the protein around region Xa located between transmembrane segments X and XI (represented as boxes) upon citrate binding. Region Xa folds back in between the two segments as a pore-loop structure. Cys-398 and Cys-414 were indicated as gray circles. Citrate (represented as an oval) binds at the membrane/cytoplasm interface. See text for further explanation.

additional evidence was presented that part of the cytoplasmic loop would form a pore-loop structure (35).

In a previous paper (27), we erroneously concluded that no binding of citrate to CitS occurs in the absence of Na⁺. Binding of substrate and co-ion would be ordered: first Na⁺ and then citrate. The conclusion was based on the lack of protection of wild type CitS against inactivation by NEM in the presence of citrate, whereas full protection was observed in the presence of Na⁺. The option that citrate might bind without protecting the protein from alkylation was considered unlikely. Here we show that citrate does bind to the transporter in the absence of Na⁺. The reactivity of Cys-398 in the SSSCS mutant was reduced upon citrate binding, especially in the inwardly oriented conformation of the transporter (Fig. 5B). The sensitivity of the wild type protein in the citrate-bound state is due to the high reactivity of Cys-414. In both orientations of the binding sites, the binding of citrate hardly changed the accessibility of Cys-414 either to NEM or MTSET (Table I).

In the alternating access model, the states with the inwardly and outwardly oriented binding sites represent the two major conformations of the transporter. The experiments revealed different properties of the two conformations of CitS. The accessibility of Cys-398 and Cys-414 was not very different in the outwardly oriented free or citrate-bound states (Fig. 4A and Table I). In contrast, in the Na⁺-bound outwardly oriented state both residues were essentially completely protected against reaction under conditions where the free transporter was fully inactivated (Fig. 7A), suggesting a significant and global change in conformation upon Na⁺ binding. At the other side of the membrane, the accessibilities of Cys-398 and Cys-414 seem to be more dependent on their local environment. Citrate binding resulted in significant protection of Cys-398 against NEM treatment (Fig. 4B) but not against MTSET, whereas little effect was observed in the case of Cys-414 against both reagents (Table I). Similarly, Na⁺ binding strongly protected Cys-398 against NEM but less so against MTSET, whereas Cys-414 was moderately protected against NEM and not against MTSET (Fig. 7B). The highly protected state of Cys-398 and Cys-414 when both citrate and Na⁺ were present (Table II) suggests a conformation different from the citrate-bound and Na⁺-bound states. Possibly, substrate and co-ions and residues near the binding sites like Cys-398 and Cys-414 are packed in a conformation that is close to the transition state of translocation.

The concentration of citrate necessary to promote the protective effect against NEM was 1 order of magnitude lower at the

periplasmic side of the membrane than at the cytoplasmic side of the membrane (Fig. 4). Under the assumption that the reactivity of the cysteine residue is similar in the two conformations of the protein, this suggests different affinities of the inward and outward facing conformations of CitS for citrate. A lower affinity for citrate at the cytoplasmic side of the membrane makes sense when the physiological function of CitS is considered. The transporter picks up low concentrations of citrate from the medium that upon translocation are released into the cytoplasm.

Two structural features are essential in the model that we propose for the translocation site of CitS (Fig. 8). (i) The Xa region of the C-terminal cytoplasmic loop folds back inbetween the transmembrane segments to form a pore-loop structure. (ii) The citrate-binding site is located at the membrane/cytoplasm interface. The latter follows from the position of Arg-428 that is believed to be one of the residues to interact with the substrate (11, 31) and is different from the situation in the LacY and GlpT structures where the substrates bind halfway up the membrane (12, 13). Important consequences of this position of the substrate-binding site may be as follows: (i) the pore is wider in the outward facing conformation than in the case of LacY and GlpT, and (ii) isomerization from the outward to the inward facing conformation may pull apart the sites on the protein that coordinate the substrate molecule, thereby disrupting the binding site. The latter mechanism would result in significant different affinities of the two states for citrate. In the model, the pore-loop containing Cys-398 and Cys-414 protrudes into the pore beyond the citrate-binding site. In the outward facing conformation, the two residues are accessible for NEM and MTSET, which are small enough to enter the pore (Fig. 8, *state a*). The relative position of the binding site and the pore-loop explains the lack of effect of bound citrate on the accessibility of Cys-398 and Cys-414; citrate binds "below" the two residues, which renders them accessible from the periplasm (Fig. 8, *state b*). Binding of Na⁺ may result in partial closure of the pore, making it too narrow for the thiol reagents to enter. In the inward facing orientation, the pore is wide enough to make the residues accessible even for AmdS (Fig. 8, *state c*). Citrate binds to the remaining interaction sites of the disrupted binding site at one side of the pore leaving enough space for MTSET to reach the two cysteine residues (Fig. 8, *state d*). Cys-398 may be closest to (part of) the citrate-binding site because it senses the presence of citrate best from both sides of the membrane. Finally, the fully loaded state is formed by closure of the pore at both sides of the membrane leaving the two cysteine residues essentially inaccessible from the water phases at both sides of the membrane. The model predicts that

a systematic scan of the accessibility of all positions in region Xa should reveal those sites that form the citrate-binding site in both orientations of the transporter and the part of the loop that forms the pore-loop structure. Such experiments are now in progress.

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